Evidence of Complete Hydrophobic Coating of Bombesin by Trifluoroethanol in Aqueous Solution: An NMR Spectroscopic and Molecular Dynamics Study

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Abstract: Bombesin is a tetradecapeptide that possesses a random coil structure in pure water. In the presence of 30% (*v*/*v*) 2,2,2-trifluoroethanol (TFE), it adopts a partial helical conformation involving the C-terminal amino acids 6–14. This conformational change, known as the TFE effect, is studied here in terms of the solvation state of the peptide at different TFE concentrations by means of intermolecular homo- and heteronuclear NOE measurements. When an aqueous solution of bombesin is titrated with TFE, a continual decrease in the water/peptide interactions and a concomitant increase in the TFE/peptide interactions is observed, and at 30% (*v*/*v*) TFE no homonuclear NOEs between water and the peptide can be detected. The conformational transition of the bombesin molecule is thus accompanied by a complete surface covering with TFE. A parallel molecular dynamics (MD) study of the peptide in aqueous solution with the single-point charge (SPC) water model and in a 30% (*v*/*v*) TFE/water mixture with a recently developed TFE model has also been performed. The 10 ns simulations were in agreement with the experimental data. The calculations indicate stabilisation of the α-helix in the H_2O/TFE mixture, in contrast to the situation in pure water, and clustering of the TFE molecules around the peptide.

Keywords: bombesin · molecular dynamics · NMR spectroscopy · solvent effects · trifluoroethanol

Introduction

The crucial functions of the proteins and peptides in living systems are based on their very specific three-dimensional structures. The folding of proteins is a highly complex process driven by many noncovalent interactions between internal residues and external surroundings. Our understanding of the folding process is still limited, although, fortunately, there are a variety of experimental strategies that provide some insight into the factors that control the folding of proteins into stable secondary and tertiary structures. It is known that external parameters such as the solvent, temperature, pH and the presence of denaturants dramatically affect the native conformations, thermodynamics and dynamics of proteins and peptides. Such changes are extensively used to extract information about the process of folding.[1]

Of particular interest is the effect of certain solvents on the conformations of peptides. Alcohols such as methanol, ethanol, 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), at different concentrations and to varying extent, induce secondary structure in some peptides.[2] TFE is very well known as one of the most efficient inducers of secondary structure (mainly, but not exclusively, helicity) in many peptides and proteins. In addition, the physical and chemical properties of an aqueous TFE solution resemble those of a biomembrane and, therefore, TFE is called a membrane-mimetic solvent. In fact, TFE has been employed to simulate a hydrophobic environment and to establish the in vivo conformations of many peptides that either reside within or are bound to membranes.[3] From a theoretical point of view, both direct[4] and indirect[5, 6] mechanisms of the TFE effect have been proposed.

As a general rule, it has been observed that a peptide dissolved in aqueous TFE attains a stable conformation at approximately 30% (*v*/*v*) TFE; no changes occur on increasing the TFE concentration further.[4] Therefore, it is reasonable to assume that TFE-induced folding processes and the solvation states of proteins are interconnected phenomena and, by investigating the solvation of a peptide in aqueous TFE, insight into the folding processes might be obtained. A battery of experimental techniques has been used to investigate both the solvation and folding of peptides and proteins (calorimetry, laser Raman and infrared spectroscopies, fluorimetry, circular dichroism, X-ray analysis, mass spectrometry);[1] NMR methods,[7] including chemical shift[8] and relaxation studies,[9] are currently used to characterise directly the

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solvation of a peptide. As a further NMR approach, the detection and evaluation of intermolecular NOEs[10] can provide spatial information about the solvation process by mapping the solute–solvent interactions.[11] However, the expected signal enhancements are small compared with intramolecular NOEs and can only be measured with difficulty in time-consuming procedures. In spite of this, the technique has been successfully employed to investigate site-specific interactions and/or preferential solvation between neutral organic molecules and solvents under neutral conditions.[12, 13]

We have recently reported the solvation state of a short peptide in aqueous TFE solutions by measuring intermolecular homo and heteronuclear NOEs.[14] Although no conformational changes occurred due to the small size of the peptide, relevant information about its solvation at different TFE concentrations was obtained. Such results encouraged us to apply the methodology to a more complex system, the conformation of which is dependent on the TFE concentration. We report herein the results of measurements of the intermolecular homo and heteronuclear NOEs between bombesin and water/TFE at different TFE concentrations. The aims of this study were to ascertain whether the conformational switch is accompanied by a change in solvation state and whether helical and unordered domains of the peptide have different solvation states.

Parallel to the experimental investigation, an atomistic approach by using MD has been undertaken to analyse theoretically the peptide solvation state and the stability of the α-helical conformation, both in water and in TFE/water mixtures. A recently developed TFE model[6] was used, which is able to accurately reproduce the activity coefficients of the mixtures.[15] This is the most important parameter for understanding the solvent/peptide interactions.[16] In binary mixtures, the activity coefficient is an important variable that provides insight into the intrinsic interactions between the two solvents. For example, from the solutions of Kirkwood buffer theory, it is possible to correlate the radial distribution function (i.e., the three-dimensional geometrical disposition of the two different solvents) with thermodynamic quantities such as partial molar volumes, isothermal compressibilities and chemical potentials (activity coefficients). If these quantities can be reproduced by a theoretical model of the solvent system, one may expect that the interactions between the different molecules (between the binary mixture and peptide) will also be adequately described.

The aim of this MD analysis was to understand the various factors inherent to the peptide–solvent interactions, such as secondary structure stability and the clustering properties of the TFE molecules around the peptide.

Bombesin belongs to a family of compounds that exhibit a variety of biological activities in numerous tissues and cell types.[17] The bombesin-like peptides exhibit a high sequence homology at the C-terminal nonapeptide, and this is responsible for their related pharmacological effects.[18] It has been postulated that, in a biological environment, the more hydrophilic N-terminal region remains in the aqueous phase exhibiting an unordered structure, whilst the relatively hydrophobic carboxyl terminus, bearing the pharmacologically active groups for triggering the biological responses, is inserted into the membrane as a helical structure.[19]

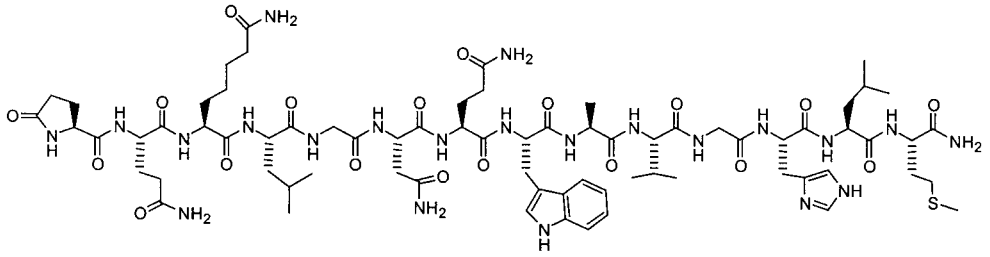
Bombesin was first isolated from frog skin and its amino acid sequence was identified as Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (Glp = pyroglutamic acid).[20] Its conformation in various solvents has been studied and it is known that it adopts an unordered structure in aqueous media and in dimethyl sulfoxide,[21] but a partial helical structure in aqueous solutions containing 30% TFE. The latter is considered to be the in vivo structure of bombesin when it is anchored to a membrane.[22]

Results and Discussion

We report herein on a one-dimensional ^{1}H NMR study of intermolecular interactions between bombesin and water and/or TFE. The assignment of the ^{1}H NMR signals as well as the structure elucidation of bombesin in water and/or TFE have been reported previously;[8, 21, 22] thus, we have concentrated our efforts on the detection and interpretation of intermolecular NOEs between bombesin and either water (homonuclear NOE) or TFE (heteronuclear NOE).

The respective one-dimensional homo- and heteronuclear NOE experiments were performed as described in our previous work on a tetrapeptide.[14] Homonuclear NOEs were successfully measured by using the modified double-pulsed field-gradient spin-echo (DPFGSE-NOE) procedure. For heteronuclear purposes, minor changes in the standard one-dimesional ^{1}H,^{19}F-HOESY sequence had to be implemented. The expected signal enhancement as a consequence of intermolecular NOEs is small and may be distorted by residual solvent signals. To better suppress such unwanted solvent signals, the use of the DPFGSE-NOE technique has been shown to be very helpful.[14]

In the one-dimensional homonuclear experiments, we had to account for the fact that inversion of the water signal



Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

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simultaneously affects the signals of both the solvent-exchangeable protons and of those H_α protons, for which the chemical shifts are identical or extremely close to that of the water signal. Therefore, the results of these measurements have to be interpreted with caution.

Titration of a sample of bombesin in water with [D_3]TFE: An 8 mM solution of bombesin in H_2O/D_2O (9:1) was examined by NMR spectroscopy and then titrated by the sequential addition (10% volume portions) of [D_3]TFE.

To identify interactions between water and bombesin, one-dimensional DPFGSE-NOE experiments were performed ($\tau_m = 190$ ms), the results of which are shown in Figure 1a. As mentioned above, not all of the observed signals necessarily arise exclusively from intermolecular NOEs between water and bombesin; they may also be due to intramolecular NOEs after saturation transfer to exchangeable amide protons. The amide protons that experience saturation transfer have the opposite signal phase. The fact that practically all the signals show NOE contacts with water indicates complete coverage of the bombesin by water and this may be correlated with the postulated unordered structure of bombesin in water, the residues of which would be statistically equally exposed to the solvent. The experiment shows that the intensities of the NOEs differ from each other to some extent, and, on the basis of our previous work, this might reflect a possible site-specific solvation of bombesin in water. However, we have not as yet recorded extensive NOE build-up curves for bombesin and therefore have to restrict ourselves to this qualitative observation.

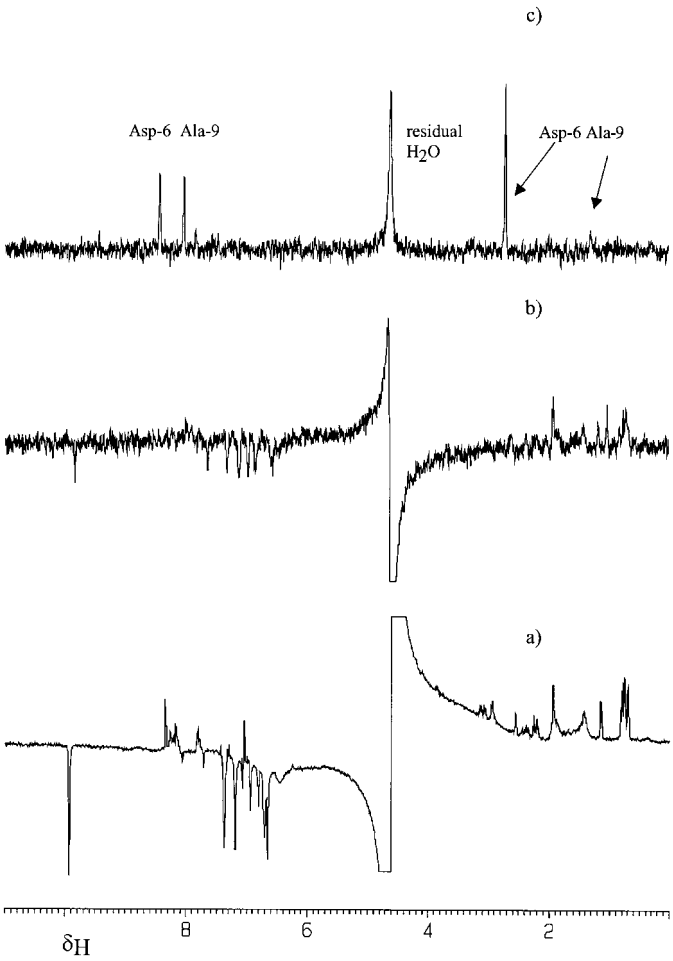

Figure 1. Homonuclear one-dimensional DPFGSE-NOE spectra showing the interactions between water and bombesin: a) bombesin in water; b) 10% TFE; c) 30% TFE; the remaining signals are due to irradiation of H_α proton of Asn-9 at the water resonance position. Spectrum c was recorded with twice as many transients as spectrum a. Note that there are chemical shift changes with increasing TFE concentration.

TFE was added portionwise to the solution of bombesin in water so as to give 10, 20, 30, 40 and 50% (*v*/*v*) of TFE in the solution. As described previously, in the presence of about 30–40% of TFE, the section of bombesin between residues 6 and 14 adopts a helical conformation, and this can be detected and followed by changes in the ^1H chemical shifts, as has been discussed in detail by Carver et al.[8] At high TFE concentrations, the existence of two well-differentiated segments has been postulated, namely a flexible and disordered N-terminus between residues 1 and 5, and a helical C-terminus between residues 6 and 14, the latter including a tighter 3_{10} helix between residues 6 and 11.[8]

Homonuclear NOE experiments were performed at each TFE concentration (10, 20, 30, 40 and 50%), and the results of two of these are shown in Figures 1b (10% TFE) and 1c (30% TFE). A concomitant diminution of the water–bombesin contacts occurred until the almost total disappearance of their signals, although, surprisingly, three very well defined NOE signals remained, irrespective of the TFE concentration. A typical spectrum is shown in Figure 1c. The signals were unequivocally identified as the corresponding amide and H_β protons of the residues Asn-6 and Ala-9. A weak signal due to the methyl group of Ala-9 can also be seen at $\delta_H = 1.30$. The explanation for the appearance of these signals is quite evident. In these 30–40% TFE solutions, the H_α signal of Asn-6 is exactly superimposed on the water signal; hence, a selective radio frequency pulse on the water signal also inverts this signal and we observe a normal intramolecular NOE to the amide proton of Asn-6 and to the $n + 3$ amino acid in the 3_{10} helix. As an experimental control, we studied the fragment of bombesin made up of residues 8–14. The same experimental procedures and conditions as employed for bombesin were applied. At 30% TFE concentration, there were no NOE contacts at all since this fragment lacks the Asn residue with its H_α proton at the water position. Furthermore, it should be noted that at this TFE concentration no saturation transfer from water to the amide protons occurs.

The observed lack of intermolecular NOEs between the tetradecapeptide bombesin or its heptapeptide fragment can only be explained in terms of a complete coating of the peptides by TFE. This is not only valid for the helical part, but also for the remaining unordered domain of residues 1–5 in bombesin. Water is replaced within the first solvation sphere over the whole peptide, irrespective of its conformational state. Therefore, we conclude that the hydrophobic interaction between TFE and the peptide is a necessary but not the only requirement for helix induction, since the N-terminus remains unordered but is also preferentially solvated by TFE.

In Figure 2 the alternative heteronuclear NOE spectra obtained from the same solutions are depicted for the

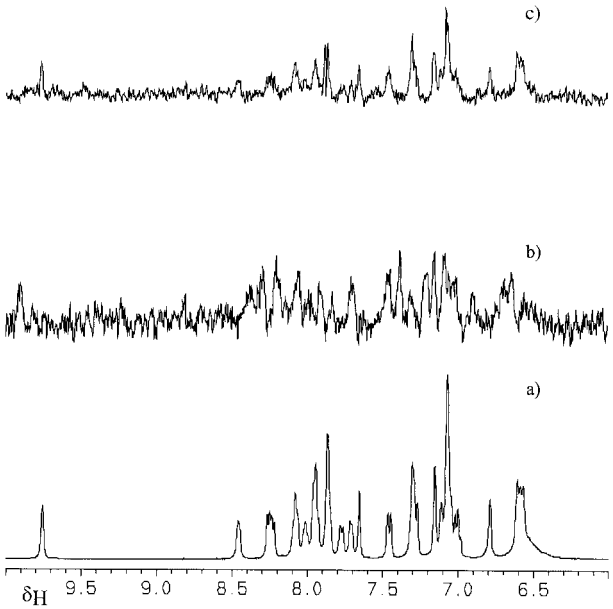

Figure 2. ^{19}F heteronuclear NOE between TFE and bombesin in water/TFE mixtures as detected by ^{1}H NMR spectroscopy in the amide/aromatic region: a) normal ^{1}H NMR spectrum; b) 10% TFE; c) 30% TFE. Note that there are chemical shift changes with increasing TFE concentration.

aromatic and amide regions. As one might expect, the higher the TFE concentration in the sample solution, the more interactions between TFE and bombesin are detected. The spectra show, as in the case of a previously studied tetrapeptide,[14], some moderate intensity variations in the signals from the individual amino acid residues, which suggests a rather homogeneous solvation of all the nuclei of the peptide responsible for the low-field resonances by TFE. We have also investigated whether varying the order of addition of the solvents has any influence on the type and strength of the bombesin–solvent interactions. For this purpose, an 8mM solution of bombesin in deuterated trifluoroethanol was analysed by NMR spectroscopy and then titrated by the sequential addition (5% volume portions) of a H_2O/D_2O mixture (9:1). In the TFE-rich region from 0 to 20% water, significant changes in the chemical shifts of both the amide proton signals and those in the aliphatic region were observed. Such changes are related to conformational changes that have not previously been described in detail for bombesin. However, identical NOE results were obtained at water concentrations higher than 20%, regardless of whether the experiments started from a high water or a high TFE concentration.

Molecular dynamics analysis: Molecular dynamics (MD) calculations with the GROMOS96 force field were performed in two solvent boxes of 1322 single-point charge SPC water molecules and 774 SPC water molecules with 91 TFE molecules, in order to assess whether the experimental findings can be reproduced by theory. The bombesin starting conformation for both the simulations (water and water/TFE mixture) was an α-helix. With no crystallographic data or NMR-averaged structure at hand, the reason for using the aforementioned initial structure was to ascertain whether the stability of the α-helical conformation is different in the presence of the TFE in the time limit of the simulations. In Figure 3, the main-chain root-mean-square deviations (RMSD) of the backbone atom positions of bombesin from the starting structure are shown in pure water (top curve) and in the TFE/water mixture (bottom curve).

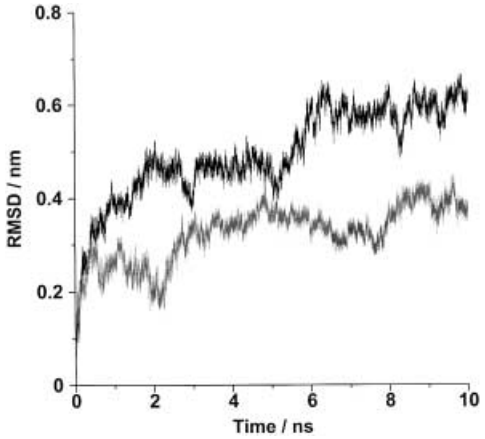

Figure 3. Results of MD calculations on the bombesin backbone in water (top) and in a 30% (*v*/*v*) TFE/water mixture (bottom). The curves give the RMSD deviations from the ideal α-helical starting structure of bombesin.

With this backbone RMSD analysis, it is possible to deduce the average displacement of the backbone atoms from the starting conformation, that is, modifications of the starting α-helix.

In the solvent mixture, the peptide is always closer to the starting structure compared to the situation in water, with a maximum deviation of 0.4 nm in the former and of 0.65 nm in the latter case. The greater stability of the α-helix in the TFE/water mixture with respect to that in neat water has thus been demonstrated.

For a better understanding of the individual contributions to the total RMSD based on the backbone atoms, an RMSD and root-mean-square fluctuations (RMSF) of the single residues may be performed. In the case of RMSF analysis, it is possible to gain information on the extent to which the individual backbone atom residues are able to "wobble" about their averaged displacement positions.

In Figure 4 the RMSD and RMSF values for each residue are displayed. In Figure 4, the RMSD values in water are seen to be large over the entire length of the peptide, with the extremities being strongly unfolded. In the mixture, the overall stability of bombesin is maintained with very low RMSD values, especially between residues 5 and 10. In Figure 4, the fluctuations in water are seen to be larger than those in the H_2O/TFE mixture. For the latter, the values are quite low over the entire length of the peptide. These data can be compared with the experimental observations: a helical conformation was detected between residues 5–14 in the mixture, while a random coil was found in aqueous solutions. Whereas experimentally the helical conformation of bombesin in TFE/water mixture is found to be located between the residues 5–14, the MD data show that the RMSD is very low between residues 5 and 10, but high between residues 11 and 14.

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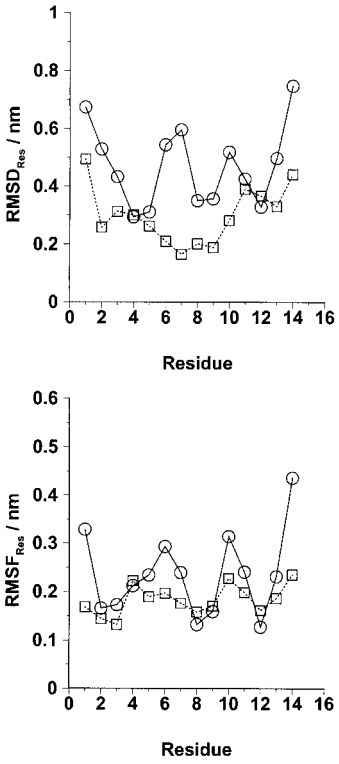

Figure 4. Calculated bombesin backbone RMSD (top) and RMSF (bottom) per residue in water (circles, solid line) and in a 30% (*v*/*v*) TFE/water mixture (squares, dotted line).

Most probably, 10 ns of simulation is insufficient for a complete folding/unfolding of the bombesin, and simulation for a longer time would be required for the N,C termini of the peptide to achieve a better agreement with the experiment. In fact, other simulations conducted on α-helix peptides have indicated conformational equilibration on a timescale of 150–200 ns.[23, 24] Nevertheless, the 10 ns used in this work can serve as a starting point for analysing the relative stability of the peptide in the two mixtures and, especially, the solvation states, for which shorter times are required.

Figure 5 shows the partial density distribution of water and TFE around the peptide. The presence of bombesin itself in the solvent box is depicted by the solid line.

The partial density distributions were obtained by dividing the cubic solvent box into ten slices, and averaging over time as viewed from the perspective of the principal axis of the α-helix. This method of calculating the box partial densities is very useful for obtaining a first clear impression of the TFE clustering over the entire peptide.

As can be seen in the figure, TFE molecules are able to cluster around the peptide, forming a high density coating

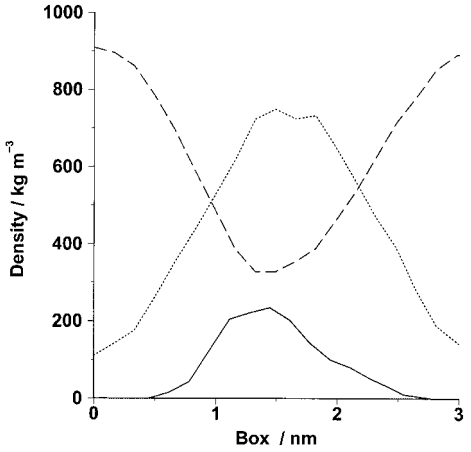

Figure 5. Calculated partial density across the solvent box for the peptide bombesin (solid line), TFE (dotted line), and water (dashed line).

(dotted line), with the amount of water reaching a minimum (dashed line). Consequently, the concentration of TFE around the peptide is higher than the starting 30% (*v*/*v*). A first rough estimate can be made, considering the maximum and the minimum density of the TFE ($\rho_{TFE} = 750\ Kg\,m^{-3}$) and water ($\rho_{SPC} = 340\ Kg\,m^{-3}$), respectively, around the peptide. With the molar volumes of TFE and SPC water in a 30% (*v*/*v*) TFE/water mixture being $V_{m(TFE)} = 0.07$ M and $V_{m(SPC)} = 0.019$ M,[15] the resulting local concentration of TFE is equal to 59% (*v*/*v*). This value is practically twice the nominal volume TFE concentration of the starting solution.

For a pictorial representation, in Figure 6 a stereoview snapshot of the bombesin in the box of TFE/water solution is given. The clustering of the TFE molecules around the peptide is evident.

Conclusion

We have shown that the experimental method employed previously for the detection of intermolecular NOEs to study the solvation of an unstructured peptide is also valid for bombesin, a peptide whose conformation is TFE dependent. In the presence of small amounts of TFE, all contacts between water and the peptide are severely diminished as a conse-

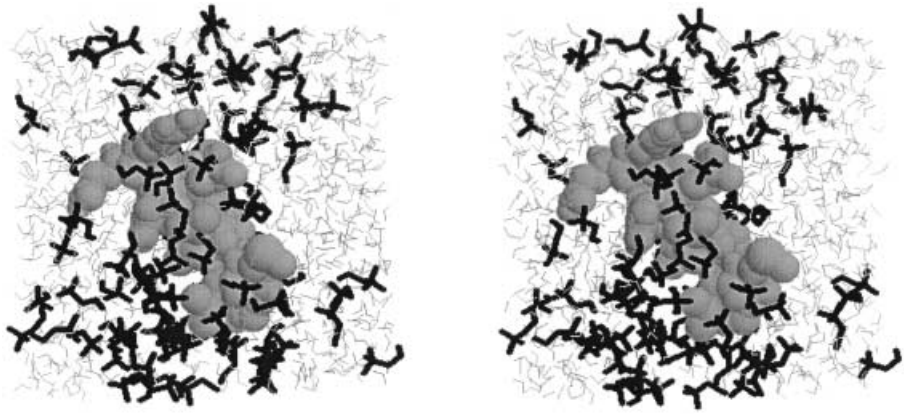

Figure 6. Stereoview of the simulation box of bombesin in a water/TFE mixture.

quence of the replacement of water molecules by TFE on the surface of the peptide. At about 30% TFE concentration, the entire peptide is preferentially solvated by TFE regardless of its conformational state. Some remaining homonuclear NOE signals are due to artifacts of the method arising from the inversion of H_α proton signals together with the water signal. The MD analysis is in agreement with the experimental data. Thus, the greater stability of the α-helix in a water/TFE mixture as compared to neat water, as detected by NMR spectroscopy, has been reproduced (in the limits of a 10 ns simulation). This phenomenon appears to be strictly correlated with the high density of TFE molecules around the peptide, which are able to create a coating with an "apparent" TFE concentration higher than the nominal starting value of the solution. We have found 59% (*v*/*v*) TFE in the vicinity of the peptide, higher than the 30% (*v*/*v*) of the bulk solution. The consequences of the experimental and computational results presented here with respect to the mechanisms of protein folding are under further investigation.

Experimental Section

Bombesin was purchased from Bachem and was used without further purification. The segment of bombesin consisting of residues 8–14 was obtained as a gift from Professor M. Rico, C.S.I.C. (Madrid, Spain). Deuterated water was obtained from Aldrich and [D_3]TFE from CIL (99% D), and both were used as received.

NMR spectroscopy: For NMR studies related to the titration of the aqueous solution of bombesin with [D_3]TFE, samples were prepared by dissolving either the peptide or its segment in 400 μL of H_2O/D_2O (9:1) containing 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as an internal reference (pH* 3.1). [D_3]TFE was added in 10% volume portions so as to give 10, 20, 30, 40 and 50% TFE solutions. The titration of a solution of bombesin in [D_3]TFE with water was performed by the sequential addition of 5% volume portions of H_2O/D_2O (9:1) so as to give 5, 10, 15, 20, 30 and 40% water solutions. In no cases were the solutions degassed.

All the NMR experiments were performed on a Bruker DRX400 spectrometer (9.4 T) at 300 K with samples in 5 mm tubes. In each case, the lock signal was adjusted to D_2O, but with the 100% [D_3]TFE sample an external reference was used to adjust the lock signal (capillary of [D_6]DMSO).

Intermolecular homonuclear NOE experiments were performed by using the pulse sequences published in ref. [14]. Measurements were made at each TFE concentration at 300 K, with a mixing time of 190 ms, a relaxation delay of 2 s and a number of transients depending on the concentration of the peptide in the sample (4 K for the more concentrated solutions; 8 K for the more dilute ones). Intermolecular heteronuclear NOE experiments were performed in a QNP probe head without gradients with a mixing time of 500 ms.

MD simulations of the bombesin helix in SPC water and TFE/SPC water mixture: NpT conditions were used in all simulations. The temperature was controlled through a weak coupling to an external temperature bath, with a coupling constant of 0.01 ps. The pressure was maintained by weak coupling to an external pressure bath of $P_0 = 1$.[25] Both the simulations in pure SPC water[26] and in the mixture with the TFE model[6] were performed with the experimental compressibility of pure water taken to be 4.5×10^{-5} bar^{-1} and with a coupling constant of $\tau_P = 1$ ps^{-1}.[25] The LINCS algorithm[27] was used to constrain all bond lengths in TFE. For the water molecules, the SETTLE algorithm was used.[28] A twin-range cut-off for the calculation of nonbonded interactions was applied. All interactions within a short range cut-off of 0.8 nm were updated after each step, whereas all interactions (Coulomb and Lennard-Jones) within a long range cut-off of 1.4 nm were updated only after every five steps together with the pair list. The cut-off values were in accord with those used as standard in the GROMOS96 force field.[29] A dielectric permittivity of $\varepsilon_r = 1$ and a time step of 2 fs were used. The starting conformation for the simulations of the peptide bombesin, for which no crystal structure is available, was taken to be an α-helix. The peptide was centred in a cubic box of dimension 3.2 nm and solvated with either pure SPC water molecules or a mixture of SPC water and TFE molecules. The protonation state of the peptide at pH 3 gives a total charge of +2. A number of Cl^- counterions were therefore added to each system to reduce the overall charge of the system. This was achieved by replacing water molecules at the most positive potential. All solvent molecules associated with any atom within 0.15 nm of the peptide were removed. The final systems contained either 1322 SPC water molecules or 774 SPC water and 91 TFE molecules (corresponding to approximately 30% (*v*/*v*)). The GROMOS96 force field was used to describe the peptide. All bond lengths in the peptide were constrained using the LINCS[27] algorithm. Before starting the simulations, the systems were first minimised for 100 steps with the steepest descent algorithm to eliminate unfavourable contacts. Initial velocities were assigned from a Maxwellian distribution corresponding to the selected temperature. To assess the relaxation and equilibration of the systems, simulations of duration 100 ps were conducted. After equilibration, simulation runs of duration 10 ns were performed for the analysis.

Acknowledgements

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